a1 cont'd

foreseeable future with transgenic plants or animals. "Crude" rGal-A from the leaf IF has a specific activity of over 1,000,000 U/mg of protein, whereas CHO, COS-1 and insect cell extracts and supernatants are maximally only 10 - 20,000 U/mg; (36,41,42)."

The paragraph begining at page 27, line 11 should read:

"We have measured the enrichment provided by the affinity resin α-galactosylamine Sepharose with a C12 arm (49). Some or all of the three effective chromatography steps were combined as necessary with a size exclusion fractionation to yield highly purified enzyme(s). Because our current source of enzyme is so enriched (Fig. 3), and several of the published purification steps we have shown to be compatible with the plant IF extracts, we anticipate no problems in enzyme purification. Pure enzyme preparations were shipped to the laboratory of Drs. Roscoe Brady and Gary Murray for evaluation of enzyme activity with ^{14}C-galactose-labeled ceramide trihexoside. These colleagues were responsible for the development of the therapeutically effective glycoform of glucocerebrosidase used to treat Gaucher disease."

a2

The paragraph begining at page 27, line 24 should read:

"We scaled up the purification of up to four candidate therapeutic enzymes as necessary in our indoor greenhouses. In our initial experiment, 38 and 48 percent of the total rGal-A activity was recovered upon the first infiltration and centrifugation treatment (Construct rGal-A12-SEKDEL) for a yield of >50 mg of enzyme per kilogram of leaf material. Experience with the extraction of glucocerebrosidase from the IF indicates that additional enzyme is recovered in a second treatment. In these experiments one leaf was collected for each sample from each of two plants. There was considerable plant to plant variation in the level of enzyme activity. We analyzed more carefully the accumulation of enzyme activity over time post-inoculation to optimize yields. Our facilities are more than sufficient to provide the 1 kilogram quantities of biomass necessary to purify nanomoles of enzyme for the following sequence and structural work. Sequence analysis and MALDI-TOF molecular weight determination was performed as a commercial service by Commonwealth Biotechnologies, Inc. N-terminal sequence is by the

a3

a3 cont'd automated Edman degradation. C-terminal sequence is by carboxypeptidase digestion followed with amino acid analysis."

The paragraph begining at page 29, line 16 should read:

a4 "Process equipment was fabricated and modified by a group of skilled vendors and craftsmen capable of fabricating specialized equipment designed by the company, and has excellent field experience working in large scale operations."

The paragraph begining at page 37, line 18 should read:

"The post-translational processing of native glucocerebrosidase (GCR) in human cells is complex. Two primary translation products are derived from two in-phase start codons. These precursors, a 2:1 mixture of 60 kDa and 57 kDa proteins, are proteolytically processed to 55 kDa as they pass into the lumen of the ER. High mannose and complex glycans are subsequently added in the ER and Golgi compartments to yield 62 and 66 kDa glycoforms. Finally, exoglycosidases generate a mature 59 kDa lysosomal enzyme. Recall that glycosylation is required for both enzymatic activity and lysosomal targeting of transfused enzyme. Sialic acid, galactose, and N-acetylglucosamine residues are enzymatically removed in vitro by the a5 sequential action of glycosidases to prepare glucocerebrosidase for therapy. The core pathway for biosynthesis and processing of N-linked complex glycans in plants appears identical to that found in animals. There are three known differences which occur later in the pathway. Sialic acid is not reported in complex glycans from plants, and the α1-3 fucose and β1-2 xylose linkages are unique (34). As analyzed by SDS/PAGE, rGCB has an apparent molecular weight of 59 kDa, and comigrates with the mannose-terminal therapeutic glycoform. We have not yet detected a significant shift in mobility upon treatment with glycosidases (PNGase F, Endo H, α 1-3 fucosidase) in our preliminary glycosylation analysis. However, the enzyme has an apparent molecular weight increase of 4 kDa over the proteolytically processed and unglycosylated form (55 kDa) and must be glycosylated for activity. Additional digestions are in progress with a more extensive set of endo- and exoglycosidases and known plant glycoprotein controls. N-Glycosidase A is reported to hydrolyze all types of N-glycan chains from glycopeptides and glycoproteins."

The paragraph begining at page 38, line 17 should read:

"The signal peptide of rGCB is processed at the correct site. A very small quantity of protein was prepared for sequence analysis by purification through Phenyl-Sepharose, ConA-Sepharose and RP-HPLC to produce a single band on SDS-PAGE comigrating with authentic glucocerebrosidase. The sequence obtained was consistent with the known sequence of processed GCR (Table 3). In this particular analysis, the first two positions were not resolved because some degradation occurred during sample preparation. Correct proteolytic cleavage of a signal peptide is also confirmed for a mouse antibody light chain molecule expressed in tobacco leaves (35).

The paragraph (the title of the table) at page 38, line 26 should read:

"TABLE 3. STRUCTURE OF THE N-TERMINUS OF rGCB".

The paragraph begining at page 39, line 13 should read:

"The results of a typical experiment are shown in Table 4. The increase in specific activity corresponds to a similar increase in the amount of cross-reacting material observed in a Western blot and is therefore not an artifact of the enzyme assay in the different fractions. Furthermore, rGCB activity was very stable in crude extracts using this particular detergent buffer. The increase in specific activity can therefore be attributed to an enrichment of rGCB in the IF relative to the whole cell homogenate. The actual concentration of rGCB in the IF is likely to be much higher, because PAGE analysis of the IF fraction shows some contamination with known cytoplasmic markers. The highest specific activity we have measured in an IF sample is 20,000 U/mg. If we assume rGCB has the same specific activity as the human enzyme (1.5 X 10^6 U/mg), this corresponds to 1.3% of the IF protein obtained by this method."

The paragraph (the title of the table) at page 39, line 25 should read:

"TABLE 4. LOCALIZATION OF rGCB TO THE INTERCELLULAR FLUID"

The paragraph begining at page 40, line 8 should read:

"High Levels of rGCB Expression in Leaf Tissue Induce Gene Silencing. The T0 individuals described in Table 4 are by definition hemizygous. They contain various loci generated from independent insertion events, having no corresponding insert on the homologous chromosome. The thirteen T0 individuals from Group A were self-pollinated and assayed for levels of enzyme expression in the T1 generation in order to analyze the effects of gene dosage (homozygotes versus hemizygotes) and to identify candidate T1 families for future seed increase. Kanamycin-resistant transgenic plants were randomly selected from segregating families and analyzed for rGCB expression. The number of probable loci was estimated by chi-square analysis of the linked kanamycin-resistant phenotype at >95% confidence level. There are several T1 families with a heritable mean rGCB activity in the range of 200-300 U/mg (nmol 4-MUG hydrolyzed per hour) in the total homogenates that we have selected for further production of the enzyme (Table 5)."

a 10

The paragraph (the title of the table) at page 40, line 21 should read:

a 11

"TABLE 5. EXPRESSION OF rGCB IN THE T1 GENERATION".

The paragraph begining at page 60, line 28 should read:

"Using the suicide substrate, conduritol ß-epoxide (CBE), inhibition of recombinant glucocerebrosidase (rGCB) activity in the presence of plant glucosidases was achieved. Enzyme activity was measured at 37°C in a reaction mixture containing 5 mM methylumbelliferyl ß-D glucoside, 0.1 M Potassium Phosphate, 0.15% Triton-X100, 0.125% sodium taurocholate, 0.1% bovine serum albumin, pH 5.9 with and without CBE. Total glucosidase activity and rGCB activity were measured by hydrolysis of the fluorescent substrate 4-methylumbelliferyl glucopyranoside. Total protein was determined using the Bio-Rad Protein Assay based on the method of Bradford (Bradford, M. Anal. Biochem. 72:248; 1976). Table 7 contains the GCB recovery data from TMV transfected plant tissue."

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The paragraph begining at page 64, line 17 should read: